DISCOVERY

Ontogeny, tissue specificity and mRNA expression changes in angiotensinogen gene upon *Aeromonas hydrophila* infection in *Puntius sarana*

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ABSTRACT

Angiotensinogen (AGT), an acute phase protein and its expression increases during inflammation. Being a constituent of Renin-Angiotensin System (RAS), AGT helps in vascular permeability, leukocyte infiltration and tissue proliferation. A partial mRNA encoding AGT gene was cloned and sequenced from the liver tissues of medium carp, olive barb *Puntius sarana* and a nucleotide sequence of 350 bp encoding 116 deduced amino acids was generated. The AGT sequences are highly conserved in fishes and the *P. sarana* AGT showed 80% and 88% similarity to *Danio rerio* AGT gene at the nucleotide and amino acid level, respectively. The distribution of the AGT transcripts in major tissues were analysed by RT-PCR and revealed the constitutive expression in brain, gill, muscle, intestine, spleen, skin and liver. The highest level of expression was marked in liver, however, the absence of the AGT transcript was noticed in anterior kidney and heart. The AGT mRNA was detected from unfertilised eggs to 21 days post fertilisation onwards indicating maternal transfer of transcripts, in *P. sarana* . Further, AGT expression level in liver of *Aeromonas hydrophila* challenged and healthy control *P. sarana* was analysed and a significant increase in expression was noticed at 1 to 12 h and again at 7 and 14 days post challenge. The current investigation further suggests the association of AGTs with bacterial infection and it is the first report on ontogeny study of this gene.

Key words: Angiotensinogen, *Puntius sarana* , Ontogeny, *Aeromonas hydrophila* infection.

Abbreviations: AGT-Angiotensinogen, Leu-Leucine, Phe-Phenylalanine

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1. INTRODUCTION

Angiotensinogen (AGT) is an alpha-glycoprotein that helps in initiation of enzyme-linked hormonal cascade known as 'Renin-Angiotensin' System (RAS). This key endocrine system is also considered as a master regulator of cardiovascular homeostasis and hydro-mineral balance in vertebrates (Crackower et al., 2002). The RAS is composed of AGT that cleaved enzymatically by renin to Angiotensin I (Ang I) and subsequently Ang I acts as a precursor to form the active Angiotensin II (Ang II). Ang II is a pluripotential octapeptide hormone with several biological actions such as vasopressure regulation, fluid osmolarity and cardiac morphogenesis (Kobayashi and Takei, 1996). Recent studies have shown that Ang II not only have a cardiovascular functions, but it also acts as an effector molecule during different stages of defence functions of body like inflammatory responses, vascular permeability, leukocyte infiltration, tissue hypertrophy/proliferation, and fibrosis (Brasier et al., 2002; Cheng et al., 2005). AGT, being the first molecule, cleaved by renin to trigger the endocrine cascade and have the ability to regulate the production of Ang II by limiting its rate of production in the RAS pathway. Recently, studies have also revealed that AGT is a crucial acute phase protein that increases during inflammation and stimulated by lipopolysaccharides. It has a direct and active role in modulation of immune responses by recruiting monocytes into the blood vessels and promoting tissue repair (Salzeta et al., 2001).

Being an active member of serine proteases, AGT is found to be is highly conserved in mammals. The recent reports have revealed the presence of AGT in teleosts and its expression profile during infection and injury (Chen et al., 2008; Umasuthan et al., 2012). In all the vertebrates, the primary organ of synthesis of AGT is mainly liver but it can be transported to the extracellular environment during need. Many workers have discussed the role of AGT in fish species like *Platichthys flesus* (Larsen et al., 2007), *Sparus sarba* (Wong et al., 2007), *Plecoglossus altivelis* (Chen et al., 2008), *Salmo salar* (Veiseth-Kent et al., 2010) and *Oplegnathus fasciatus* (Umasuthan et al., 2012) and evidences also confirmed the localisation of this gene in different tissues of fish body (Brown et al., 2000). Among the widely distributed cyprinids, the olive barb, *Puntius sarana* (Hamilton) is a commercially important and highly preferred fish species of India and other Asian countries (Ghosh et al., 2005). However, according to report of National Bureau of Fish Genetic and Resources (NBFGR), Lucknow, India, on current status of fish species (NGFGR report, 2011), olive barb is in threshold of extinction and categorized under 'vulnerable species'. The reduced riverine conditions and outbreak of diseases has pushed the fish into the verge of extinction. Since few years, efforts have been made towards the successful breeding, culture and production of the particular species, but diseases are still a main concern for their vulnerability (Jena et al., 2008). Hence, immunity towards diseases and the exploration of immune factors involved in the immunity of this fish species are highly essential in the present scenario. Though many workers have defined the role of angiotensinogen in teleosts and its function in immunity, an attempt was made to characterize AGT in *P. sarana* and its tissue-specific distribution. A study was also carried out to determine its mRNA expression during early embryogenesis and the response of AGT towards a bacterial pathogen *Aeromonas hydrophila* infection in sarana species.

2. MATERIALS AND METHODS

2.1. Fish

Healthy *P. sarana* juveniles weighing 30 g were collected from the farm of the farm of the CIFA, Kausalyaganga, and Bhubaneswar, India. The fish were killed with overdose of anaesthesia (MS222) and the liver tissues were collected immediately in RNA*later* (Ambion) for further study.

2.2. RNA extraction and cDNA synthesis

Total RNA from liver tissues (~50 mg) was isolated using TRI reagent (Sigma, USA) according to manufacturer's instruction and treated with DNAse I (Fermentas, USA), to minimise the chances of genomic DNA contamination. Further, the purity and quantity of RNA was checked spectrophotometrically by taking $OD_{260\ nm}/OD_{280\ nm}$ using NanoDrop ND1000, (NanoDrop Technologies Inc., USA). For cDNA synthesis, one microgram of total RNA was incubated with random hexamer primer (100 µM) for 5 min at 70 °C and then cooled at 25 °C for 10 min to anneal the primer properly to the RNA. To the mixture, MMLV-RT buffer (10X), dNTP (100 µM), RNAse inhibitor (10U/µL)and MMLV-RT enzyme (200 U/µL) were added, and incubated for 5 min at 25 °C, followed by 42 °C for 1 h and 95 °C for 2 min in a thermal cycler (Master cycler Gradient Eppendorf, USA). RT-reactions lacking reverse transcriptase (RT minus) but not RNA were also performed to verify that the samples did not contain genomic DNA.

2.3. PCR amplification of the gene

Before going for AGT amplification, the integrity of the RNA samples was checked by RT-PCR using primer pairs of house-keeping gene, β-actin (F-5'GAC TTC GAG CAG GAG ATG G-3' and R-5'CAA GAA GGA TGG CTG GAA CA-3') and for AGT gene, specific self-designed primer pairs were used (Angio F- 5'-ATGTCCACCCATTCAACCTC-3' and R-5'-TGAGTCCTAACAGCTGCTGG-3'). The PCR

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reaction for β-actin and AGT gene consisted of 40.70 µL nuclease-free water, 5 µL (10X) PCR buffer, 1 µL (10 mM) dNTPs, 1 µL (10 pico mole) of each forward and reverse primers for each gene, followed by addition of 0.3 µL (1.5 U/ µL) Taq DNA polymerase (Genei, India) and 1 µL of cDNA. The amplification schedule includes an initial denaturation of 94 °C for 3 min, followed by 28 cycles of denaturation of 94 °C for 45 sec, then an annealing at 45 sec (55.3 °C for β-actin and 45.7 °C for AGT)and extension at 72 °C for 1 min 30 sec, followed by a final extension for 10 min at 72°C. Finally, the samples were run in 1% agarose gel to get the amplicons.

2.4. Cloning and sequencing of AGT cDNA

The amplified PCR product of AGT was purified using Genei purification kit and cloned into pGEMT vector (Promega) according manufacturer's instruction. Plasmid DNA containing the target gene was isolated by alkaline lysis method and insertion of the gene was confirmed by restriction digestion analysis. Afterwards, positive clones were purified following phenol-chloroform extraction method and three clones were outsourced for sequencing.

2.5. Ontogeny and tissue-specificity of AGT transcript

For ontogeny study, three pairs of sexually matured male and female *P. sarana* (150-200g) were obtained from the monoculture pond of Central Institute of Freshwater Aquaculture and administered with 'Ovaprim' for breeding. The breeding was performed separately in the indoor hatchery and fishes started releasing eggs after 6 h and hatched after 18 h of injection. To check the appearance of AGT at different stages of development, unfertilized eggs, fertilized eggs and hatchlings were collected in RNA*later* (Sigma) separately at 0, 1, 3, 6, 9, 12, 18, 24 h, 48 and 4, 7, 15 and 21 days post-fertilization. For, tissue-specificity study, samples of nine different organs such as brain, gill, heart, muscle, intestine, spleen, skin, liver and anterior kidney were collected in RNA*later* from three apparently healthy juveniles (100-200 g) after anaesthetization and all the samples collected for gene expression analysis was kept at -70 ^{0}C for further analysis.

The samples were subjected to RNA extraction, quality and quantity checks, and cDNA synthesis as mentioned in detail in section 2.2. Further, the integrity of the RNA samples was checked by RT-PCR using β-actin primers. Only good quality RNA samples from three number of each stage/ tissue were subjected to AGT-specific PCR (as described in section 2.3) for ontogeny and tissue specific expression analysis. The obtained nucleotide and deduced amino acid sequences were analyzed using the BioEdit Sequence Alignment Editor (7.0.5.3) (Hall, 1991) and the resulted final sequence was subjected to nucleotide BLAST query at NCBI to get information about the related species and their sequence identity. Further, the confirmed sequences for this species were deposited in GenBank to obtain accession number (accession no.-JX010735).

2.6. Expression of AGT in *P. sarana* infected with *Aeromonas hydrophila*

2.6.1. Fish

Apparently healthy *P. sarana* juveniles of average weight 150.3 ± 8.2 g were obtained from a monoculture pond of the Institute and maintained in the wet laboratory in 500L capacity FRP tanks for acclimatization before the challenge experiment. During these periods the animals were fed with standard pellet feed at 3% of body weight and water was siphoned daily to maintain the proper water quality by removing faecal materials and unused feed particles. During the study, the optimal water quality was maintained (temperature 29 ^{0}C-31 ^{0}C, dissolved oxygen: 5.65 ± 0.72 mgL^{-1}; pH: 8.2 ± 0.82; nitrites: 0.015 ± 0.009 mg L^{-1}; ammonia: 0.109 ± 0.024 mg L^{-1}).

2.6.2. Preparation of bacteria

The pathogenic strain of *A. hydrophila* (Ah # 04/2003) collected from the Fish Health Management Division of CIFA was cultured for 18 h in tryptone soya broth at 30 ^{0}C and the harvested cells were washed and resuspended in phosphate buffer saline (PBS). The concentration of bacteria was checked by measuring the OD at 540 nm and LD_{50} dose (2.24 ×10^7 CFU/mL) was calculated following Reed and Muench (1938).

2.6.3. Experimental design for challenge test

To carry out the challenge test, seventy-five fishes distributed randomly in to three fibre-reinforced plastic tanks (500L capacity) and infected with 100 µl of *A. hydrophila* in PBS by intraperitoneal injection (at a pre-determined LD_{50} dose of 2.24 ×10^7 CFU/mL). The control group fish (ten numbers kept in one FRP tank) were injected with 0.1 ml of PBS only. For the analysis of AGT immune gene, liver tissues from infected fish were collected aseptically in RNA*later* at 0, 1, 3, 6, 12, 24, 48 h and 4, 7, 10 and 14 d post injection taking one fish from each tank (in triplicate) for each time period and kept for RNA extraction, and further expression analysis. RT-PCR for AGT expression was performed as described earlier taking three samples for each time periods. The PCR products were run

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in 1% agarose gel and the level of expression was analysed densito-metrically using AlphaEase_FC Imaging Software (Alpha Innotech Corp., USA) and significant expression level was calculated taking ratio of AGT gene/ β-actin product.

2.6.4. Statistical analysis

The significant ($p < 0.05$) differences in expression pattern of the target gene relative to β-actin in control and infected fish was determined using Mann Whitney *U*-test. Mean ± SE of gene was calculated from three samples taken for each time period.

3. RESULTS

3.1. Sequence analysis of AGT gene

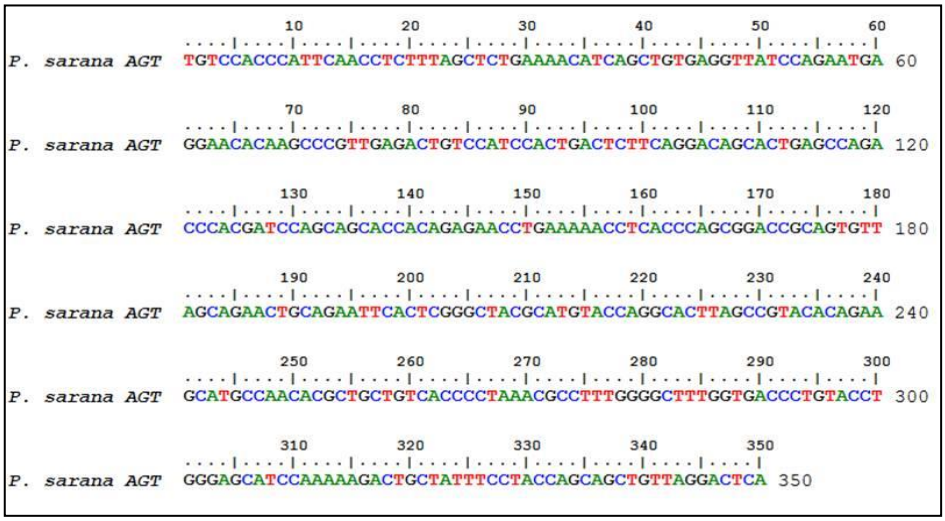

Figure 1
The nucleotide sequence of *Puntius* AGT cDNA

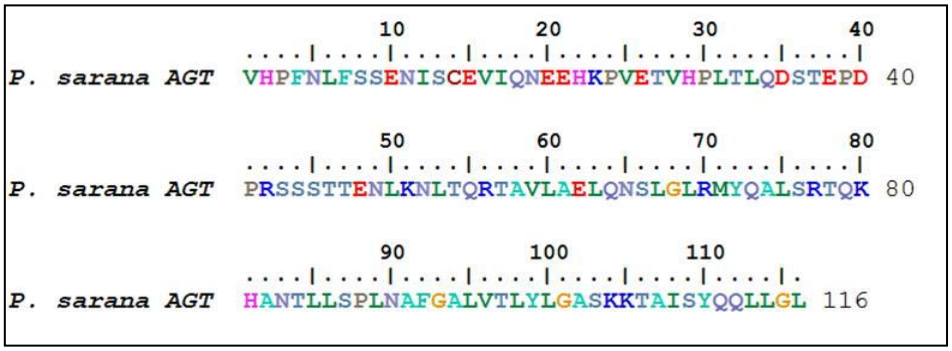

Figure 2
The deduced amino acid sequence of *Puntius* AGT mRNA

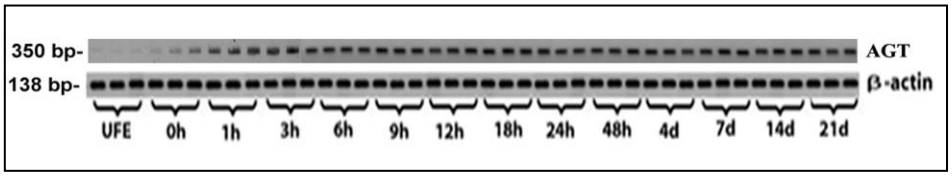

Figure 3
Ontogenic appearance of AGT transcript in *P. Sarana*

As the primer was designed from the short conserved sequences of AGT gene, a partial mRNA sequence of AGT was generated for *P. sarana* and the amplicon was found to be of 350 bp (GenBank accession no: JX010735) encoding 116 deduced amino acids. During BLAST analysis, the sequence revealed highest similarity with *Danio rerio* AGT showing 80% homogeneity (Figs. 1 & 2). The deduced amino acid sequence of AGT showed the highest similarity with *D. rerio* (88%) and structural similarity from the BLAST analysis confirmed that this peptide belongs to the serpin superfamily. Although this Leu-rich (Leu-16.4%) short peptide sequence

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contains no signal peptide (as predicted from ProtParam program of EXPASy Molecular Biology Server), but a cleavage site for renin was noticed in the N-terminal sequence (Leu[6] and Phe[7]) in *P. sarana,* indicating the transcript as the precursor form of angiotensin I.

3.2. Ontogeny and tissue-specificity study of AGT

The ontogenic expression showed the appearance of AGT from unfertilised eggs onwards and remained constant up to 21 days post fertilisation (Fig. 3). The AGT transcripts were detected in most of the tissues viz., brain, gill, intestine, muscle, spleen, skin showing expression in liver tissue. However, the anterior kidney and heart tissues were absent for AGT mRNA transcript during tissue-specificity study (Fig. 4).

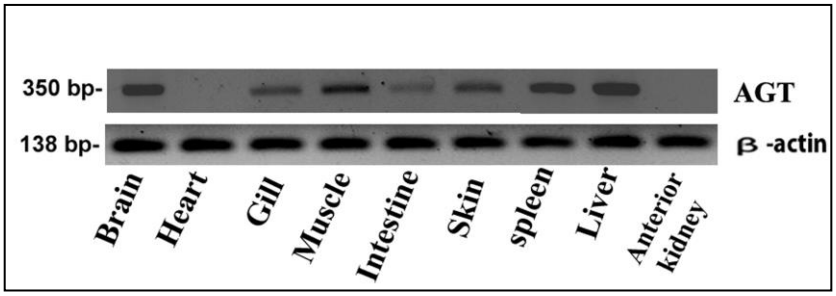

Figure 4
Tissue-specific expression of AGT transcript in different organs *P. Sarana*

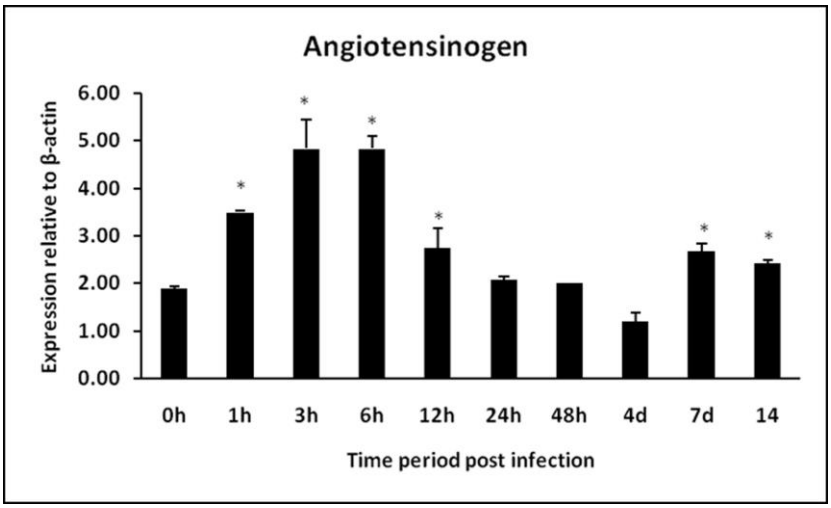

Figure 5
Expression profile of AGT transcripts in different time period post- infection in *P. sarana* infected with *A. hydrophila*

3.3. Expression of AGT in *P. sarana* during *A. hydrophila* infection

Upon infection with *A. hydrophila*, a significantly high level of expression of AGT was observed from 1 to 12 h post infection and returned back to control fish level at 24 h to 4 days post infection (dpi). However, the expression was again up-regulated significantly on day 7 and 14 pi (Fig. 5).

4. DISCUSSION

The partial mRNA sequence of AGT of 350 bp was determined in this study in *P. sarana* . The obtained partial sequence was highly conserved for all the fish species and showed sequence similarities of 80%, 70%, 69% with zebra fish, ayu and rainbow trout, respectively, which is in accordance to earlier reports (Watanabe et al., 2009; Umasuthan et al., 2012).The full gene information has been described in few other fish species like ayu (accession no: AM921805) and zebra fish (accession no: BC095585).

The transcripts were present from unfertilised eggs stage onwards to 21 days post fertilisation during this study, suggesting the maternal transfer of transcripts and the increased intensity of bands within 1 hpf indicated immediate synthesis of these transcripts in *P. sarana*. The early synthesis of this gene product might be playing role in defence as well as embryogenesis of vasculature and seems to be first report in fish ontogeny study. In human and chicken eggs, angiogenesis is a regular process where new blood cells

formed from earlier vasculature and recently it has also been reported from zebra fish (Paul et al., 1993; Leung et al., 2006). However, very little is known about the presence of AGT gene in eggs and larvae of other fishes including carps. Being a part of angiogenesis and RAS system, the AGT plays an important role in blood regulations and blood vessel formation. Hence, its expression from unfertilised to fertilised eggs and adults in *P. sarana* might indicate the function of RAS in angiogenesis during aquatic stress, inflammation-related disorders and stress related hypertension.

In olive barb, the AGT transcript was found to be present in various examined tissues including brain, gill, intestine, muscle, spleen, skin, liver, indicating the presence of local RAS in these organs. However, the transcript was not detected in anterior kidney and heart. Liver, being the major site for synthesis of AGT, showed its intense presence in this species also. According to mammalian systems, the existence of circulatory RAS has been identified in several organs, including heart, brain, kidney, and digestive organs (Paul et al., 2006). Similarly, AGT was identified in brain, liver, kidney and intestine of silver sea bream and brain, spleen, liver, kidney and intestine of ayu (Chen et al., 2008; Umasuthan et al., 2012). Working on expression of AGT during *A. hydrophila* infection, a significant up-regulation the gene was observed at earlier time periods (1-12 h post infection) in infected *P. sarana* showing the involvement of angiotensinogen during early infection and inflammation. The septicaemia caused by the bacteria might have lead to loss of blood volume during the initial time periods. It is also well reported that the RAS can be activated when there will be drop in blood pressure leading to increase in production of angiotensinogen and it was obvious that the infection of *A. hydrophila* might have resulted an inflammatory reaction in the infected tissues. Further, the reduction in the expression level of AGT at 12 hpi to 4 dpi, indicated the inability of liver to produce enzyme due to infection related damage of the organ. Again the up-regulation of the gene at 7 and 14 dpi onwards suggested the regeneration of liver tissue in survivors of infection but pathogens might not be fully eliminated from the system. Similar higher expression level of AGT was noticed in various tissues of *P. altivelis* against *A. hydrophila* and rock sea bream upon induction with *Edwardsiella tarda* (at 6h to 48 h pi), LPS (at 12 h post injection) and during all time periods against *Streptococcus iniae* (Chen et al., 2008; Umasuthanet al., 2012). Although, the expression of AGT transcript is essential to the activation of RAS, further enhancement of role and bioactivity of RAS need due investigation. All of these earlier studies and current findings collectively suggest that hepatic AGT transcription is associated with bacterial inflammation.

SUMMARY OF RESEARCH

1. This study provides the first evidence of AGT gene in a medium carp fish *P. sarana* and showed a close relationship with other fish species.
2. The study further confirms the presence of AGT transcripts from unfertilised egg onwards up to fry stage and detected in most of the organs except heart and kidney.

FUTURE ISSUES

Further work is needed to explore the full gene sequence of AGT and its modulation during various infection processes.

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Conflict of Interest

The authors declare no conflicts of interests any matter related to this paper.

Data and materials availability

All related data have been presented in this paper.

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